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PASTEURELLA MULTOCIDA FROM OUTBREAKS OF AVIAN CHOLERA IN WILD AND CAPTIVE BIRDS IN DENMARK

K. Pedersen,^{1,3} H. H. Dietz,¹ J. C. Jørgensen,¹ T. K. Christensen,² T. Bregnballe,² and T. H. Andersen¹

¹Danish Veterinary Institute, Department of Poultry, Fish, and Fur Animals, Hangøvej 2, DK-8200 Aarhus N, Denmark

²National Environmental Research Institute, Department of Wildlife Ecology and Biodiversity, Grenåvej 12, DK-8410 Rønne, Denmark

³Corresponding author (email: kpe@vetinst.dk)

ABSTRACT: An outbreak of avian cholera was observed among wild birds in a few localities in Denmark in 2001. The highest mortalities were among breeding eiders (*Somateria mollissima*) and gulls (*Larus* spp.). Pulsed-field gel electrophoresis (PFGE) was conducted using *ApaI* and *SmaI* as restriction enzymes and restriction enzyme analysis (REA) using *HpaII*. The *Pasteurella multocida* subsp. *multocida* strain isolated from birds in this outbreak was indistinguishable from a strain that caused outbreaks in 1996 and 2003. Most isolates from domestic poultry had other PFGE patterns but some were indistinguishable from the outbreak strain. Among 68 isolates from wild birds, only one PFGE and one REA pattern were demonstrated, whereas among 23 isolates from domestic poultry, 14 different *SmaI*, 12 different *ApaI*, and 10 different *HpaII* patterns were found. The results suggest that a *P. multocida* strain has survived during several years among wild birds in Denmark.

Key words: Avian cholera, cormorant, eider, *Pasteurella multocida*, pasteurellosis.

INTRODUCTION

Pasteurella multocida is a Gram negative, oxidase positive, non-motile, non-spore forming, facultative anaerobic rod-shaped or coccoid bacterium. The species is divided into three subspecies: *P. multocida* subsp. *multocida*, *P. multocida* subsp. *gallicida*, and *P. multocida* subsp. *septica*, mainly on the basis of ability to ferment dulcitol and sorbitol (Mutters et al., 1985). The majority of isolates from wild birds belong to the subspecies *multocida*, followed by subsp. *gallicida*, whereas the subsp. *septica* constitutes only a minor fraction (Hirsh et al., 1990; Snipes et al., 1990).

Pasteurella multocida has an impressive host spectrum, including numerous mammals and birds. Other authors have reported differences in virulence for various hosts and from strain to strain but this is poorly understood (Snipes et al., 1988; Rimler and Glisson, 1997; Petersen et al., 2001).

Among poultry, *P. multocida* is the cause of avian cholera, a disease that has been described almost worldwide and causes great losses to the poultry industry. The disease can be treated with antimicrobials

or prevented with vaccines. In wild bird populations, *P. multocida* has been reported as causing infection in over 100 species; it is likely that most or all species are susceptible under certain circumstances. In some species, including domestic poultry, the disease may develop into epizootics involving hundreds or thousands of birds (Botzler, 1991; Rimler and Glisson, 1997).

In domestic poultry, healthy carriers and chronic forms of the infection are well described (Rimler and Glisson, 1997; Muhairwa et al., 2000). In wild bird populations, it is unusual to observe clinically diseased birds due to a rapid and fatal development of the disease. The infection is a bacterial septicemia and, due to the rapid onset and progression, the birds are usually in good nutritional condition (Mensik and Botzler, 1989). There may be petechial hemorrhages in internal organs, liver necrosis, mucoid enteritis, and mucoid discharge from the nares (Botzler, 1991; Rimler and Glisson, 1997).

Bird species have different susceptibility to the disease, but the course of an outbreak depends on other factors as well. When outbreaks involve more than one species, there tends to be sequential mor-

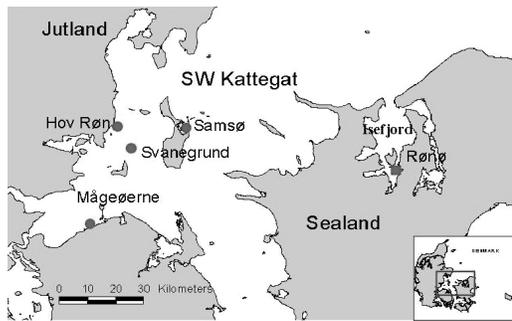


FIGURE 1. The location of common eider colonies with documented outbreaks of avian cholera in Denmark. Colonies affected in both 1996 and 2001 are shown as circles, the colony only affected in 2001 is shown as a square.

tality and some species suffer disproportionately higher mortality than others. A high population density is a predisposing factor as probably is stressful weather, such as precipitation and sudden change from hot to cold weather (Botzler, 1991).

This paper describes an outbreak of avian cholera among wild birds in Denmark in 2001 and compares *P. multocida* isolates with isolates from other wild birds and domestic poultry using pulsed-field gel electrophoresis (PFGE) and restriction enzyme analysis (REA).

MATERIALS AND METHODS

Bird populations under study

During April and May 2001 dead and dying eiders (*Somateria mollissima*) were found in breeding colonies in southwest Kattegat and in Isefjord, Denmark (Fig. 1). Some of these birds as well as dead individuals of other species were collected for examination. *Pasteurella multocida* subsp. *multocida* was isolated from all of these birds by standard bacteriologic techniques except for one great cormorant (*Phalacrocorax carbo*) from Mågeøerne. Thus, avian cholera was diagnosed as the primary cause of mortality among birds from these areas.

For comparison, isolates of *P. multocida* from outbreaks among wild birds in 1996 and 2003 were included in this study together with isolates from domestic poultry and wild birds, farmed in captivity for hunting purposes.

Bacterial isolates and culture conditions

Primary cultures from dead birds submitted to the laboratory were grown on blood agar

(Blood agar base No. 2 [Oxoid, Greve, Denmark], supplemented with 5% calf blood) incubated overnight aerobically at 37 C. Subcultures were made on blood agar. Isolates were identified using API 20NE strips (bioMérieux, Marcy l'Etoile, France) supplemented with tests for oxidase, catalase, mannitol (all subspecies positive), sorbitol (subsp. *multocida* and *gallicida* positive), and dulcitol (subsp. *gallicida* positive), and inspection of colony morphology. Cultures were freeze dried and stored at room temperature. A total of 91 isolates from various birds were selected for further investigation. Of these, 48 were from the outbreak of avian cholera among wild birds in 2001 and, for comparison, eight isolates from an outbreak in 1996 (see Christensen et al., 1997) and 11 from an outbreak in 2003 were selected from our laboratory's culture bank together with one isolate from an eider from 1997. Finally, 23 isolates from domestic poultry and one turkey and captive pheasants (*Phasianus colchicus*) and partridges (*Perdix perdix*), from throughout the country isolated during 1997, 1998, 2001, and 2002 were used (Table 1). The isolates from domestic poultry were collected from diseased birds submitted to our laboratory where a diagnosis of avian cholera had been made. Further information about these isolates was usually not available.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis was carried out essentially as described (Nauerby et al., 2000). Briefly, strains of *P. multocida* were grown overnight in brain heart infusion broth at 37 C to an optical density (OD)₆₂₀ of approximately 0.5 and then approximately 1.5 ml of culture was used for DNA preparation essentially as described by Cameron et al. (1994). Slices of agarose embedded DNA were digested with *Sma*I or *Apa*I. The fragments were separated in a 1% agarose gel (Pulsed Field Certified Agarose, Bio-Rad, Hercules, California, USA) in 0.5×TBE buffer by using a CHEF-DR III system (Bio-Rad). The electrophoresis conditions were 6 V/cm at 12 C for 20 hr. The ramping times were 1–30 sec for 22 hr for *Apa*I and 0.5–40 sec for 22.5 hr for *Sma*I. Following electrophoresis, the gel was stained in aqueous ethidium bromide (BioRad), 2 µg/ml, for 15 min, destained in water for 15 min, and photographed in 254 nm ultraviolet light. In addition to *Apa*I and *Sma*I, the four enzymes *Kpn*I, *Bam*HI, *Eag*I, and *Xba*I were tested on five isolates.

Restriction enzyme analysis

The DNA used for PFGE investigations was also used for REA. Slices of the agarose em-

TABLE 1. Pulsed-field gel electrophoresis (PFGE) and restriction enzyme analysis (REA) patterns of isolates of *Pasteurella multocida*.

| Year of isolation | Bird species | Subspecies | Number of isolates | <i>Sma</i> I PFGE profile | <i>Apa</i> I PFGE profile | <i>Hpa</i> II REA profile |
|-------------------|--|------------------|--------------------|---------------------------|---------------------------|---------------------------|
| 1996 | Eider (<i>Somateria mollissima</i>) | <i>multocida</i> | 8 | A | 1 | H1 |
| 1997 | Eider | <i>multocida</i> | 1 | A | 1 | H1 |
| | Hen | <i>multocida</i> | 1 | B | 2 | H2 |
| | Duck (domestic) | <i>septica</i> | 1 | C | 3 | H3 |
| 1998 | Hen | <i>multocida</i> | 2 | A | 1 | H1 |
| 2001 | Cormorant (<i>Phalacrocorax carbo</i>) | <i>multocida</i> | 18 | A | 1 | H1 |
| | Eider | <i>multocida</i> | 17 | A | 1 | H1 |
| | Gull (<i>Larus</i> spp.) | <i>multocida</i> | 10 | A | 1 | H1 |
| | Duck (wild, species not recorded) | <i>multocida</i> | 1 | A | 1 | H1 |
| | Heron (<i>Ardea cinerea</i>) | <i>multocida</i> | 1 | A | 1 | H1 |
| | Merganser (<i>Mergus serrator</i>) | <i>multocida</i> | 1 | A | 1 | H1 |
| | Hen | <i>gallicida</i> | 1 | D | 4 | H1 |
| | Hen | <i>multocida</i> | 1 | E | 5 | H4 |
| | Duck (domestic) | <i>multocida</i> | 1 | F | 6 | H5 |
| | Pheasant (<i>Phasianus colchicus</i>) (farmed) | <i>multocida</i> | 1 | G | 7 | H1 |
| | Partridge (<i>Perdix perdix</i>) (farmed) | <i>multocida</i> | 1 | A | 1 | H1 |
| | Turkey (domestic) | <i>multocida</i> | 1 | H | 8 | H6 |
| | Hen | <i>multocida</i> | 2 | I | 9 | H7 |
| | Hen | <i>septica</i> | 1 | J | — | H1 |
| | Hen | <i>multocida</i> | 2 | A | 1 | H1 |
| | Hen | <i>multocida</i> | 1 | K | 10 | H1 |
| 2002 | Hen | <i>multocida</i> | 2 | A | 1 | H1 |
| | Pheasant (farmed) | <i>multocida</i> | 2 | L | 11 | H9 |
| | Pheasant (farmed) | <i>multocida</i> | 2 | M | 1 | H9 |
| | Hen | <i>multocida</i> | 1 | N | 12 | H10 |
| 2003 | Eider | <i>multocida</i> | 4 | A | 1 | H1 |
| | Gull (<i>Larus</i> spp.) | <i>multocida</i> | 5 | A | 1 | H1 |
| | Oyster catcher (<i>Haematopus ostralegus</i>) | <i>multocida</i> | 1 | A | 1 | H1 |
| | Mute swan (<i>Cygnus olor</i>) | <i>multocida</i> | 1 | A | 1 | H1 |

bedded DNA were prepared as for PFGE, except that *Hpa*II was used for digestion of the DNA following the instructions of the manufacturer (New England Biolabs, Beverly, Massachusetts, USA). Fragments were separated by electrophoresis in 1% agarose gels containing ethidium bromide, 4 μ l of a 10 mg/ml solution per 300 ml gel. Electrophoresis was carried out in 1 \times TAE buffer for 18–20 hr at 40 volt, and gels were photographed in ultraviolet light. A *Hind*III digested λ -phage (Molecular-Weight Marker II, Roche, Basel, Switzerland) was used as fragment size marker. Restriction enzyme analysis patterns were given arbitrary designations H1–H10.

RESULTS

Description of the 2001 outbreak of avian cholera

Mortality among the breeding eider females was highest at Rønø (88.2%; 1,359

dead, 181 alive and incubating), lower at Samsø (81.5%; 150 dead, 34 alive and incubating) and lowest at Hov Røn (49.9%; 412 dead, 413 alive and incubating). Eiders that had died from pasteurellosis were also recorded in two other colonies in southwest Kattgat: Svanegrunden (16 dead) and Mågeørne (15 dead). The small numbers of dead eiders recorded at Hov Røn and Samsø compared to the number recorded at Rønø may be related to these colonies having been reduced by approximately 80% and 95%, respectively, during an outbreak of avian cholera in 1996 confined only to breeding colonies in southwest Kattgat (Christensen et al., 1997).

In contrast to the situation during the outbreak of pasteurellosis in 1996, a number of great cormorants breeding at Samsø were found dead (95 dead, 2,958 nests). Dead cormorants that died of pasteurellosis were also recorded at Svanegrunden (57 dead, 1,395 nests), Mågeørne (nine dead, 1,848 nests), and Rønø (four dead, 75 nests), but not in any other of the 44 surveyed cormorant breeding colonies. Dead gulls were found in lower numbers in all surveyed colonies where eiders had died from pasteurellosis, mostly herring gulls (*Larus argentatus*) and great black-backed gulls (*Larus marinus*).

Pulsed-field gel electrophoresis results

Pulsed-field gel electrophoresis patterns obtained with *ApaI* as the restriction enzyme yielded 10–15 usually clear and well separated bands. Twelve different *ApaI* patterns were distinguished while a single isolate belonging to the subspecies *septica* could not be digested with this enzyme (Fig. 2a). *SmaI* generated considerably more bands, rendering patterns more difficult to interpret. *SmaI* yielded 14 different banding patterns (Fig. 2b). Results of the five isolates that were tested with *KpnI*, *BamHI*, *EagI*, and *XbaI* showed that isolates that were indistinguishable with *SmaI* or *ApaI* also were indistinguishable with these enzymes, and those that were different with *KpnI*, *BamHI*, *EagI*, and *XbaI* were also different with *SmaI* or *ApaI*.

For all isolates, except three, there was agreement between the *ApaI* and *SmaI* results. Two of these isolates had the unique *SmaI* pattern M but the common *ApaI* pattern 1, whereas the third isolate had a unique *SmaI* pattern but was not digested with *ApaI*. The result for this strain was therefore inconclusive in this respect (Table 1).

All isolates from wild birds, irrespective of species and year of isolation, had indistinguishable profiles, referred to as *ApaI* pattern 1 and *SmaI* pattern A. This pattern was also recognized in seven isolates from

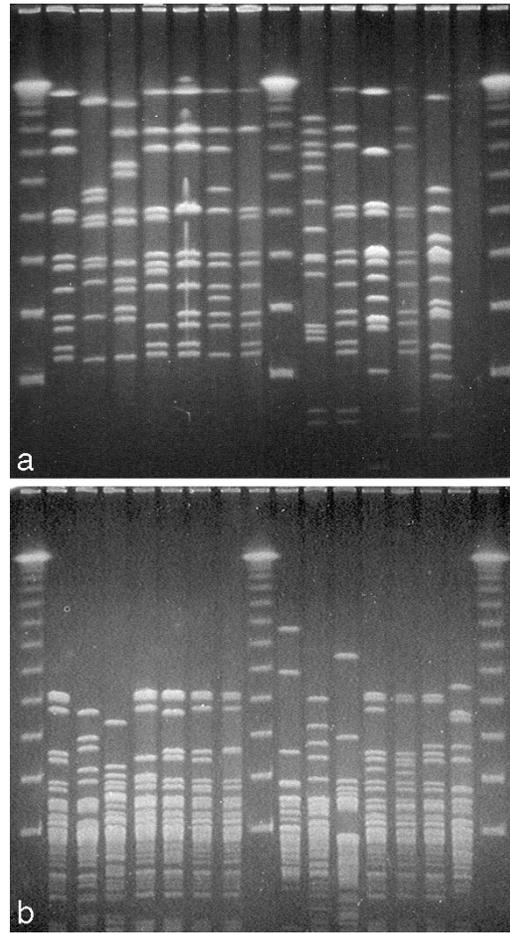


FIGURE 2. Pulsed-field gel electrophoresis patterns of *Pasteurella multocida* isolates from wild birds compared to isolates from domestic poultry using *ApaI* (a) and *SmaI* (b) as the restriction enzymes. *ApaI* and *SmaI* PFGE patterns were given arbitrary designations 1 through 12 and A through N, respectively. (a) Lanes 1, 9, and 16, PFGE molecular weight marker; lane 2–8, pattern 1–7; lane 10, pattern 8; lane 11, pattern 10; lane 12, pattern 9; lane 13, pattern 11; lane 14, pattern 12; lane 15, DNA not digested. (b) Lanes 1, 9, and 17, PFGE molecular weight marker; lanes 2–8, pattern A–G; lanes 10–16, patterns H–N.

farmed birds, i.e. six hens and a partridge. However, the PFGE patterns of the isolates from farmed birds were far more diverse: among 23 isolates, 14 different *SmaI* patterns and 12 *ApaI* patterns were recognized.

Two isolates from domestic poultry belonged to the subspecies *septica* and the PFGE patterns of these two isolates were

clearly distinct from those of the subspecies *multocida* isolates (Fig. 2a, b).

Restriction enzyme analysis results

A total of ten REA patterns were distinguished (Table 1). Isolates that were indistinguishable with PFGE were also indistinguishable with REA, and those that were identical PFGE were also identical with REA. However, a few isolates that were identical with REA could be differentiated with PFGE. Thus, REA was less discriminatory than PGFE.

DISCUSSION

Poultry farmers may be concerned that wild birds may transmit pasteurellosis to their poultry. Christensen et al. (1998) found a close relationship between wild avian cholera outbreak strains and isolates from backyard poultry. Using ribotyping these authors found that some isolates differed in the position of only a single band. They concluded that this close relationship could indicate an exchange of strains between wild birds and domestic poultry. On the other hand, Hirsh et al. (1990) and Snipes et al. (1990) concluded that *P. multocida* strains from wildlife animals were very unlikely to cause disease among domestic turkeys, although their conclusions were drawn from phenotypic traits and did not involve DNA fingerprinting techniques. Muhairwa et al. (2001) found, by comparing typing results of isolates from free-ranging poultry, dogs, and cats that, in spite of close contact between the poultry and the cats and dogs, exchange of strains between these animals was not common. The results of our study does not exclude that such exchange of strains may take place, because the PFGE pattern of the strain from wild birds was also the most common pattern among isolates from farmed birds. However, our results do not allow conclusions as to the direction or extent to which such exchange may occur. Most findings of *P. multocida* in Denmark are from backyard poultry which have access to outdoor facilities. On at least one

occasion an indistinguishable genotype was isolated from wild birds and backyard poultry during a pasteurellosis outbreak (Petersen et al., 2001). Olsen and Wilson (2001) were in favor of the theory that wild carnivores such as raccoons (*Procyon lotor*) and foxes (*Vulpes vulpes*) were the main sources of *P. multocida* for free-range turkeys. When comparing pairs of isolates from the same farm collected from outbreaks the same year, these authors found that in 70.3% of the cases the pairs of isolates had identical DNA fingerprints, indicating that in these cases the same strain persisted. In contrast, for pairs collected in two consecutive years or with a longer interval, in 63.6% and 26.7% of the cases the isolates were identical, indicating that in these cases, new strains were often introduced. The authors suggested that this could be explained by some level of movement of the wild carnivores, thereby bringing in new clones of *P. multocida*.

It is interesting that all isolates from wild birds displayed indistinguishable PFGE patterns, irrespective of species, geographic origin, and year of isolation. This indicates that *P. multocida* from avian cholera among wild birds in Denmark is clonal whereas several clones exist among domestic poultry. The fact that all wild isolates from 1996 to 2003 were indistinguishable, may indicate a remarkable genetic stability of this strain. Genetic stability of *P. multocida* has hitherto not been studied in any detail; however, Wilson et al. (1995a) found isolates with the same genetic profile over extended periods. Thus, one profile was found during 1979–83, a second profile from 1979–89, and a third from 1983–93. Because these profiles characterized the vast majority of isolates from this period, these results indicated that relatively few clones were responsible for the majority of mortality.

Hirsh et al. (1990) suggested that strains of *P. multocida* isolated from ducks were different from those isolated from geese. Our results did not indicate the presence of species-specific clones. However, strains

vary in virulence to various species. Petersen et al. (2001) found that a strain from an outbreak of pasteurellosis among wild birds was highly virulent for pheasants, partridges, and turkeys, but not for chickens. This observation raises questions about the role of wild birds as a source of infection for domestic poultry. Wilkie et al. (2000) found differences in virulence to chickens among five strains of *P. multocida*. Whether the clonal lineages found among domestic poultry in the present study are virulent for wild birds is not known, because we have no facilities to carry out virulence tests on wild bird species.

Christensen et al. (1998) concluded that the strain of *P. multocida* responsible for the outbreak was spread by migratory birds. Although this may have been true, other possibilities may be more likely. Some authors have suggested that the most important reservoir of *P. multocida* is birds that have survived an infection and act as healthy carriers. That eiders may be carriers of pasteurellosis is supported by the fact that Korschgen et al. (1978) isolated *P. multocida* from one of 236 apparently healthy eiders collected during the winter season, and from one of 357 apparently healthy nesting eider females. However, these authors did not test the virulence of the isolates. Samuel et al. (1997) isolated *P. multocida* from a healthy lesser snow goose (*Chen caerulescens*) and verified that this isolate was pathogenic to Pekin ducks. Further evidence of the lesser snow goose as a reservoir for *P. multocida* was presented by Samuel et al. (1999) who found healthy seropositive birds. *Pasteurella multocida* survives in water and soil for long periods of time and they are probably important for transmission during avian cholera outbreaks whereas in the long term they are considered less important than healthy carriers (Botzler, 1991).

During outbreaks of avian cholera in 1996 and 2001 in Denmark, unsuccessful attempts were made to isolate *P. multocida* from brackish water and sediment close to

or at the eider colonies. Thus, during the 2001 outbreak, samples of water from small, stagnant brackish water ponds at Hov Røn (four ponds), Samsø (19 ponds), and Rønø (one pond) were collected, but *P. multocida* was not recovered from any of these water samples (unpubl. data).

Previous studies of *P. multocida* from poultry have mostly relied on phenotypic characteristics (biotype, plasmids, serotype, capsule type) and there has been a need for typing methods based on genetic properties. During the last few years, several DNA based typing methods have been used on *P. multocida* isolates but unfortunately, due to methodologic differences, comparison of results is difficult. Snipes et al. (1989) used an rRNA probe (ribotyping) and REA to distinguish strains. Wilson et al. (1995a) used REA with *HhaI* as a restriction enzyme to type a collection of *P. multocida* and they suggested that REA should be used for typing of *P. multocida*. In their study of *P. multocida* from raptors (Wilson et al., 1995b), they used *HhaI* and *HpaII* as restriction enzymes, but they only found a few different patterns. Christensen et al. (1998) used *HhaI* and *HpaII* REA in combination with ribotyping. Fusing et al. (1999) used capsular typing, plasmid profiling, phage typing, and ribotyping in an investigation of toxin-producing *P. multocida* isolated from pigs and concluded that ribotyping using *HindIII* was very useful. In another study of *P. multocida* from pigs, Lainson et al. (2002) used random amplified polymorphic DNA (RAPD) typing and *AvaI* PFGE, and found PFGE to be the most discriminative typing method. Gunawardana et al. (2000) typed 95 Australian and Vietnamese isolates of *P. multocida* from cases of pasteurellosis in chickens and turkeys, using repetitive extragenic palindromic sequence polymerase chain reaction (REP-PCR) and *ApaI* PFGE and these authors were able to distinguish related and unrelated outbreaks. The authors found good correlation between the two methods but seemed to prefer REP-

PCR due to its rapidity. Boerlin et al. (2000) used *Hind*III and *Eco*RI ribotyping and *Sma*I and *Sma*I PFGE to investigate a human case of meningitis in a baby caused by *P. multocida* and implicated the cat as a potential source of the bacterium. Aalbæk et al. (1999) used REA, ribotyping, and PFGE to investigate an outbreak of hemorrhagic septicemia in fallow deer (*Dama dama*). These authors found that ribotyping, PFGE, and *Hpa*II REA categorized all isolates from the deer to a unique clone, whereas *Hha*I REA was not able to distinguish the strain from this outbreak from a strain isolated from fallow deer in the UK. In our study, we applied PFGE using *Sma*I and *Apa*I as restriction enzymes and REA using *Hpa*II. PFGE yielded more patterns than REA and REA added no further information to the PFGE results. On the basis of the results, we consider this method superior to REA, ribotyping, RAPD, and REP-PCR. Restriction enzyme analysis is difficult to interpret due to the large number of bands, whereas ribotyping displayed low diversity (Christensen et al., 1998) and is more time consuming unless the technique is automated by using a RiboPrinter. Pulsed-field gel electrophoresis can to some extent be standardized, yielding results that are comparable between different laboratories. This is more difficult for RAPD and REP-PCR. *Apa*I and *Sma*I yielded, with few exceptions, congruent PFGE results, but the banding patterns generated by *Apa*I were easier to interpret (Fig. 2), and we therefore recommend use of this enzyme. Pulsed-field gel electrophoresis is the preferred technique for typing several bacteria and we have had excellent experience for typing and epidemiologic investigation of a variety of organisms including *Streptococcus bovis* (Pedersen et al., 2003), *Pseudomonas aeruginosa* (Hammer et al., 2003), *Campylobacter jejuni* (Pedersen and Wedderkopp, 2003), and *Clostridium perfringens* (Nauerby et al., 2003).

Although avian cholera may occur throughout the year, most cases occur dur-

ing the winter and spring (Botzler, 1991). The present outbreak took place during the breeding period. Both eiders and cormorants are colonial breeding birds that occur in high densities during breeding compared to the non-breeding season. This aggregation of birds probably facilitated transmission leading to conspicuous epizootics. Indeed, previous outbreaks of pasteurellosis among eiders have only been reported at breeding colonies (Gershmann et al., 1964; Reed and Cousineau, 1967; Korschgen et al., 1978; Swennen and Smit, 1991; Christensen et al., 1997).

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